Apoptosis Kit

HT TiterTACS™Apoptosis Detection Kit

Catalog Number: 4822-96-K

Quantitative assay for the detection of apoptosis in cells.

96 Well Colorimetric Kit

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The TACS® Apoptosis Detection Kits allow for the identification of apoptotic cells. For many cell types in culture, identification of apoptosis can be achieved using a combination of morphological criteria, extraction and analysis of DNA, *in situ* detection of DNA fragmentation in immobilized cells measuring the activation of ICE-like proteases, detection of Annexin binding at the cell surface, or activation of poly-ADP ribose polymerases. However, in many cases, the treatment and analysis of a large number of samples is inconvenient.

PRINCIPLE OF THE ASSAY

The HT TiterTACS Apoptosis Detection Kit allows the quantitative colorimetric analysis of a large number of cell samples using a 96-well plate format. The assay provides the quantitation of apoptosis in cultured cells without direct counting of labeled cells. This reagent-based kit is designed for quantitation of apoptosis in suspension and monolayer cell cultures.

TACS-Nuclease™, provided in the kit, allows positive controls to be generated for each experimental system: a brief treatment of cells with TACS-Nuclease prior to labeling generates DNA breaks in every cell, providing an appropriate positive control specific for the system under study. The HT TiterTACS Apoptosis Detection Kit has a 96 well plate format that offers:

- A sensitive and easy to follow assay.
- Cost-effective screening method for up to 96 different samples.
- Quantitative read-out, allowing for statistical analysis.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

Wear gloves and exercise caution when handling formaldehyde solutions.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Cytonin™	4876-05-01	6 mL	Store at 2-8 °C.
10X TdT Labeling Buffer	4817-60-02	20 mL	
10X TdT Stop Buffer	4817-60-03	20 mL	
Strep-HRP	4800-30-06	30 µL	
Blue-Strep Diluent	4800-30-12	7.5 mL	
TACS-Sapphire™	4822-96-08	10 mL	
TACS-Nuclease™ Buffer	4800-30-16	1.5 mL	
Proteinase K	4800-30-01	2 vials (50 µL/vial)	Store ≤ -20 °C.
TdT dNTP Mix	4810-30-04	35 µL	
TdT Enzyme	4821-96-05	35 µL	
50X Mn^{2+}	4810-30-14	2 vials (50 µL/vial)	
TACS-Nuclease™	4800-30-15	15 µL	

OTHER MATERIALS REQUIRED

Equipment:

- 1- 20 µL, 20-200 µL pipettes
- 37 °C incubator
- 50 mL and 300 mL graduated cylinders
- ≤-20 °C and 2-8 °C storage
- Microplate reader
- Centrifuge and microscope adapters

Reagents:

- 10X Phosphate Buffered Saline (PBS)
- 37% Formaldehyde
- Xylenes
- 30% hydrogen peroxide
- 100% ethanol (or denatured alcohol)
- Methanol
- 5% phosphoric acid or 0.2 M HCl
- Tween® 20
- Distilled water

Disposables:

- 6 well microplate
- Microcentrifuge tubes
- 1-200 µL pipette tips
- 10 mL serological pipettes
- 15 mL tubes

REAGENT PREPARATION

The volumes given for each reagent are based on processing samples in a 96-well plate. If conical well plates are used, the volume of solution may be decreased to 30 µL per well.

Reagents marked with an asterisk (*) should be prepared immediately before use.

1X PBS - Approximately 100 mL of 1X PBS is used to process 96 samples. Dilute 10X PBS to 1X using distilled water. Store 1X PBS at room temperature.

1X PBST - For 1X PBST, add 0.05% Tween 20 to 1X PBS and mix thoroughly by gentle inversion.

***3.7% Buffered Formaldehyde -** 20 mL of fixative is used to process 96 samples.

Reaction Component	Volume
1X PBS	45 mL
37% Formaldehyde	5.0 mL
Final Volume	50 mL

Note: *Wear gloves and exercise caution when handling formaldehyde solutions.*

***Proteinase K Solution** - 50 µL of Proteinase K Solution is used per sample. Thaw Proteinase K on ice or at room temperature and then place on ice. Prepare immediately before use. To prepare add:

Reaction Component	1 Sample	100 Samples
Distilled water	50 µL	5 µL
Proteinase K	1.0 µL	100 µL

Cytonin - Cytonin is ready to use. If required, 50 µL of Cytonin is used per sample. Store at 2-8 ˚C. Discard if cloudy.

***3% Hydrogen Peroxide Solution** - 5.0 mL of 3% Hydrogen Peroxide Solution is necessary per 100 samples. To prepare 6.0 mL of solution mix:

Reaction Component	Volume
30% hydrogen peroxide	0.5 mL
Methanol	5.5 mL

1X TdT Labeling Buffer - 25 mL of 1X TdT Labeling Buffer is enough to process 96 samples. Dilute 10X TdT Labeling Buffer to 1X using distilled water. Leave at room temperature until use. Remove an aliquot of 50 µL of 1X TdT Labeling Buffer per sample to prepare the Labeling Reaction Mix and place on ice.

REAGENT PREPARATION *CONTINUED*

***Labeling Reaction Mix** - Thaw TdT dNTP Mix at room temperature and then place on ice. To maintain optimal activity, remove the TdT Enzyme from the freezer to pipette the required volume. Alternatively, place the TdT Enzyme in a ≤ -20 ˚C freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare one sample without the enzyme. To prepare 50 µL per sample:

Reaction Component	100 Samples	n Samples
1X TdT Labeling Buffer	5.0 mL	n x 50 µL
TdT dNTP Mix	35 µL	n x 0.35 µL
50X Mn^{2+}	100 µL	n x 1.0 µL
TdT Enzyme	35 µL	n x 0.35 µL

1X TdT Stop Buffer - 20 mL of 1X TdT Stop Buffer is used to process 96 samples. Dilute 10X TdT Stop Buffer to 1X using distilled water. Leave at room temperature until use.

***Strep-HRP Solution** - Prepare Strep-HRP Solution by diluting Strep-HRP 1:1250 in Blue-Strep Diluent. 50 µL of Strep-HRP Solution is used per sample.

Reaction Component	100 Samples	n Samples
Blue-Strep Diluent	5.0 mL	n x 50 µL
Strep-HRP	4.0 µL	n x 0.04 µL

TACS-Sapphire - TACS-Sapphire is ready to use. Use substrate solution at room temperature, add 100 µL of solution per well. Protect from light.

TACS-Nuclease Solution - 50 µL of TACS Nuclease Solution is required for each nuclease-treated control sample. Avoid repeated freeze-thaw of TACS-Nuclease. To prepare add:

Reaction Component	1 Sample	n Samples
TACS-Nuclease Buffer	50 µL	n x 50 µL
TACS-Nuclease	1.0 µL	n x 1.0 µL

Stop Solution - Prepare 5% phosphoric acid or 0.2 M HCl. Add 100 µL of 5% phosphoric acid or 0.2 M HCl per well to stop the colorimetric reaction.

ASSAY PROTOCOL

This section includes instructions for sample preparation, *in situ* labeling, and plate reading. The assay protocol for labeling is in tabulated form and details the steps involved in the labeling reaction. Prior to labeling, the samples must be fixed and washed in PBS. The labeling procedure begins with samples in PBS regardless of the fixation and immobilization method.

SAMPLE PREPARATION AND FIXATION

PREPARATION OF SUSPENSION CELLS

Cells grown in suspension, or prepared from dissociated tissues, can be fixed in batch solution and then transferred to 96-well plates. Alternatively, cells may be grown and fixed directly in the 96-well plates.

Batch Method

1. Harvest cell suspension by centrifugation at 500 x g for 5 minutes at room temperature. Prepare enough cells for your assay. Between 2 x 10^4 - 1 x 10^5 cells/well is recommended.
2. Wash in 1X PBS and centrifuge at 500 x g for 5 minutes.
3. Discard PBS and resuspend at 1 x 10^6 cells/mL in 3.7% Buffered Formaldehyde. Let stand for 7 minutes at room temperature (do not leave longer than 10 minutes).
4. Centrifuge at 500 x g for 5 minutes at room temperature and discard fixative.
5. Wash once with 1X PBS and centrifuge at 500 x g for 5 minutes.
6. Post-fix sample in 100% methanol for 20 minutes at room temperature.
7. Wash cell pellet twice in 1X PBS. Between washes centrifuge cells at 500 x g for 5 minutes.
8. Resuspend cells at 1 x 10^6 cells/mL in 1X PBS.
9. Distribute cells at 2 x 10^4 - 1 x 10^5 cells/well. The exact cell number should be determined empirically.
10. Proceed to Labeling Procedure.

In Well Method

1. Distribute or grow 2 x 10^4 - 1 x 10^5 cells/well in a 96-well plate.
2. Centrifuge plate at 1000 x g for 3 minutes at room temperature and discard media.
3. Wash once with 1X PBS at room temperature.
4. Fill wells with 3.7% Buffered Formaldehyde. Let stand for 7 minutes at room temperature (Do not leave longer than 10 minutes).
5. Centrifuge plates at 1000 x g for 3 minutes at room temperature and discard fixative.
6. Wash cells with 1X PBS.
7. Post fix sample in 100% methanol for 20 minutes at room temperature.
8. Wash wells twice with 1X PBS. Centrifuge between washes.
9. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *CONTINUED*

Monolayer Method

1. Centrifuge plate at 1000 x g for 3 minutes at room temperature and discard the media.
2. Wash two times with 1X PBS at room temperature. Between washes centrifuge plate at 1000 x g for 3 minutes.
3. Fill wells with 3.7% Buffered Formaldehyde. Let stand for 7 minutes at room temperature (Do not leave longer than 10 minutes).
4. Centrifuge plate at 1000 x g for 3 minutes.
5. Wash twice with 1X PBS at room temperature. Between washes centrifuge plate at 1000 x g for 3 minutes.
6. Post fix sample in 100% methanol for 20 minutes.
7. Wash wells two times with 1X PBS. Between washes centrifuge plate at 1000 x g for 3 minutes.
8. Proceed to Labeling Procedure.

LABELING PROCEDURE

STEP	INSTRUCTIONS	Notes
1	Add 50 µL of Proteinase K Solution per well and incubate for 15 minutes at room temperature.	Samples can also be treated with 50 µL/well of Cytonin as an alternative to Proteinase K. Cytonin is recommended for cells in monolayer.
2	Centrifuge plate at 1000 x g for 3 minutes at room temperature and discard the buffer.	
3	Wash once with 200 µL/well of distilled water.	Centrifuge plate between each wash.
4	Generate a positive control using TACS-Nuclease Solution.	Add 50 µL of TACS-Nuclease Solution to each control well. Incubate for 10-60 minutes at 37 °C. Other samples may be covered with PBS during preparation of the positive nuclease-treated control.
5	Wash samples for 2 minutes in 1X PBS.	Centrifuge plate between each wash.
6	Quench endogenous peroxidase. Add 50 µL 3% Hydrogen Peroxide Solution and incubate for 5 minutes at room temperature.	Do not exceed 5 minutes.
7	Wash once with 200 µL/well of distilled water.	Centrifuge plate between each wash.
8	Add 150 µL/well of 1X TdT Labeling Buffer. Leave for 5 minutes.	
9	Centrifuge plate and discard buffer.	
10	Add Labeling Reaction Mix (50 µL/well) and incubate at 37 °C for 1 hour.	Use a humidity chamber or a microplate cover during incubation.
11	Add of 1X TdT Stop Buffer (150 µL/well) for 5 minutes to stop labeling reaction.	
12	Centrifuge plate and discard buffer.	
13	Wash samples twice with 1X PBS for 2 minutes per wash.	Centrifuge plate between each wash.
14	Add Strep-HRP Solution (50 µL/well) and incubate at room temperature for 10 minutes.	
15	Wash samples four times with 200 µL/well of 1X PBST.	Centrifuge plate between each wash.
16	Add TACS-Sapphire (100 µL/well) at room temperature.	If working with suspension cells, ensure that cells are resuspended.
17	Incubate at room temperature for 30 minutes **in the dark.**	Follow kinetics of the reaction at 630 nm to determine linear range.
18	Stop reaction with 5% phosphoric acid or 0.2 M HCl (100 µL/well). Measure absorbance at 450 nm.	Read plate within 30 minutes of acid addition.

CONTROLS

The controls that should be included, especially when performing the protocol for the first time, are listed below. Controls should be performed in duplicates or triplicates.

TACS Nuclease - Treated Control - Treat two or three samples with TACS-Nuclease to generate DNA breaks. The TACS Nuclease treated controls will confirm that the permeabilization and labeling reaction has succeeded. The information can help optimize the conditions for the labeling procedure. The colorimetric readings obtained with this control will be higher than the experimental values and will provide a maximum value.

Unlabeled Control Sample - The TdT Enzyme should be omitted from the Labeling Reaction Mix for two or three samples. These controls will indicate the level of background labeling associated with non-specific binding of the Strep-HRP. These controls should have low or negligible absorbance.

Negative Control Sample - An appropriate negative control should be included in each experiment and will depend upon the system under study. Typically the negative control will be an untreated sample or normal cells. Many normal or untreated cells and tissues will have a small number of apoptotic cells, resulting in a low level of labeling.

DATA INTERPRETATION

Duplicate or triplicate samples will allow statistical validation of results. Positive and negative controls are important in data interpretation and allow optimization of *in situ* detection of apoptosis without expending valuable test samples. Refer to the Troubleshooting Guide for information if the Controls do not provide the expected result.

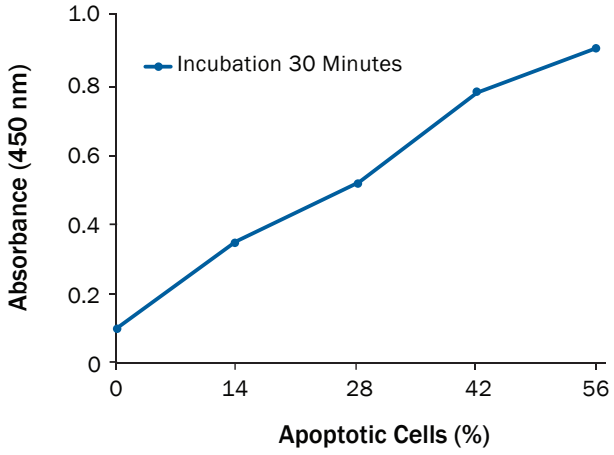

Figure 1: Quantitation of Apoptosis in Staurosporine-treated ML-1 cells using HT TiterTACS Apoptosis Detection Kit. Detection of apoptosis in fixed ML-1 cells after treatment with 1 mM staurosporine for 24 hours. Cells were harvested, fixed, and labeled according to the protocol. Cells were incubated with TACS-Sapphire substrate and the colorimetric reaction was stopped with 0.2 M HCl after 30 minutes. The percentage of apoptotic cells in the culture was estimated by enumeration using the TACS 2 TdT-DAB *in situ* Labeling Kit. The cell culture was diluted with a non-apoptotic cell culture to obtain the different concentration of apoptotic cells for the assay.

DATA INTERPRETATION *CONTINUED*

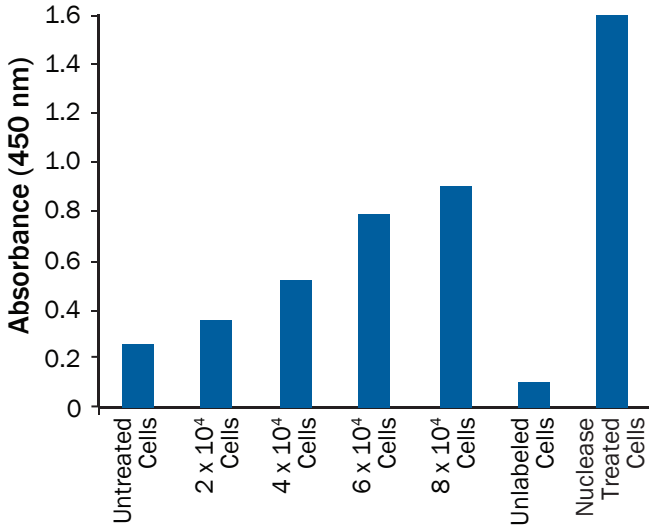

Figure 2: Quantitation of Apoptosis in Staurosporine Treated ML-1 Cells using HT TiterTACS Apoptosis Detection Kit. Data obtained after stopping the reaction with 5% phosphoric acid, 30 minutes after addition of substrate. Control included wells that were untreated (without apoptosis inducer), unlabeled (without TdT Enzyme), and nuclease-treated. All the control wells contained 100,000 cells.

Note: *Experimental results may vary depending on the type of cells, cell treatment, incubations and storage conditions. Morphological observation of the cells is recommended prior to assay.*

TROUBLESHOOTING GUIDE

STEP	INSTRUCTIONS	Notes
No labeling in TACS-Nuclease treated sample.	Poor permeabilization and/or excessive fixation with cross-linking fixative preventing enzyme access.	Optimize Proteinase K treatment or optimize time in Cytonin. Reduce time in fixative to 5 minutes.
	No DNA left in sample due to hydrolysis (poor storage of samples).	
	Excessive (removed all DNA) or inadequate nuclease treatment.	Optimize time for nuclease treatment (5 minutes up to 2 hours).
	TdT Enzyme is inactive. The enzyme is the most labile component in the kit.	TdT Enzyme must be stored at ≤ -20 °C. Do not bring enzyme up to ice temperature. Place in ≤ -20 °C block or remove aliquot from tube directly in freezer. Store in manual defrost freezer.
No labeling in experimental sample.	No apoptosis (or necrosis) occurring in sample.	If all controls gave the expected results and were processed at the same time as the experimental sample there may be no DNA fragmentation in cells within the sample. Always examine the morphology of cells.
Excessive background in negative control.	Residual unlinked Strep-HRP.	Wash cells at least 4 times with 1X PBST.
	Non-specific binding of Strep-HRP.	Incubate Strep-HRP with a blocking reagent such as 5% (w/v) non-fat dried milk or fetal bovine serum in 1X PBST.
Poor duplicate or triplicate values.	Non-sufficient centrifugation or poor removal of buffer.	Centrifuge after every wash. Use care when pipetting off buffer.
	Loss of cells after or during washes.	Use conical 96-well plate to perform assay on suspension cells. Transfer cells to flat bottom plate after incubation with TACS-Sapphire.

REFERENCES

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